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Biology and identity of fish spermatogonial stem cell

Samyra Maria dos Santos Nassif Lacerda, Guilherme Mattos Jardim Costa, Luiz Renato de França *

Laboratory of Cellular Biology, Department of Morphology, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais 31270-901, Brazil

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ABSTRACT

Although present at relatively low number in the testis, spermatogonial stem cells (SSCs) are crucial for the establishment and maintenance of spermatogenesis in eukaryotes and, until recently, those cells were investigated in fish using morphological criteria. The isolation and characterization of these cells in fish have been so far limited by the lack of specific molecular markers, hampering the high SSCs biotechnological potential for aquaculture. However, some highly conserved vertebrate molecular markers, such as Gfra1 and Pou5f1/Oct4, are now available representing important candidates for studies evaluating the regulation of SSCs in fish and even functional investigations using germ cells transplantation. A technique already used to demonstrate that, different from mammals, fish germ stem cells (spermatogonia and oogonia) present high sexual plasticity that is determined by the somatic microenvironment. As relatively well established in mammals, and demonstrated in zebrafish and dogfish, this somatic environment is very important for the preferential location and regulation of SSCs. Importantly, a long-term *in vitro* culture system for SSCs has been now established for some fish species. Therefore, besides the aforementioned possibilities, such culture system would allow the development of strategies to *in vitro* investigate key regulatory and functional aspects of germline stem cells (ex: self-renewal and/or differentiation) or to amplify SSCs of rare, endangered, or commercially valuable fish species, representing an important tool for transgenesis and the development of new biotechnologies in fish production.

1. Introduction

Spermatogenesis is a highly organized process that includes mitotic, meiotic and post-meiotic cells that ultimately leads to the sperm formation. Spermatogonial stem cells (SSCs) are the foundation of spermatogenesis and crucial for transmitting the genetic information to the next generation (Nagano and Yeh, 2013; Valli et al., 2014; Yoshida, 2012). In addition, according to studies in mammals and fish, SSCs are unique stem cells that can also be reprogrammed into pluripotent cells with the ability to differentiate into somatic tissues (Conrad et al., 2008; Oatley and Brinster, 2012; Thoma et al., 2011), rendering SSCs as a potential alternative for embryonic stem (ES) cells. Moreover, in combination with transplantation techniques, which are also available for fish (Lacerda et al., 2010, 2013a; Majhi et al., 2009; Morita et al., 2012; Nóbrega et al., 2010; Okutsu et al., 2006), SSCs could be powerful vectors for modified genes (Honaramooz and Yang, 2011).

Based mainly on the available literature for mammals, there are strong evidences that the regulation of SSC activity occurs within a specific microenvironment in the testes, the SSC niche, which may be influenced by several testis elements such as Sertoli, Leydig and peritubular myoid cells, basement membrane, and other cellular components/factors from the intertubular compartment (Caires et al., 2010; Chiarini-Garcia et al., 2003; Hofmann, 2008; Nóbrega et al., 2010; Phillips et al., 2010). Although SSC present very high biotechnological potential in aquaculture, in fish the isolation and characterization of these cells have been so far limited by the lack of specific molecular markers. Therefore, several research groups are currently investigating potential markers for early spermatogonia and, up to date, some proteins have been identified in different spermatogonial populations in distinct fish species (Bosseboeuf et al., 2013; Lacerda et al., 2013b; Nagasawa et al., 2012; Ozaki et al., 2011).

In the subsequent sections, we will review the current knowledge of SSCs morphology, kinetics, plasticity, distribution and niche in the fish testis, as well as to compile the available information related to fish spermatogonial phenotypical characterization. Also, whenever possible, we will make a comparison between the current knowledge regarding the SSCs biology in fish and

* Corresponding author. Fax: +55 (31) 34092780.
E-mail address: lrfranca@icb.ufmg.br (L.R. de França).

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mammals (mainly rodents) that are better investigated in this aspect.

2. Spermatogonial cells in fish

Spermatogenesis is sustained by SSCs, which have the potential for both self-renewal and differentiation into cells committed to sperm production (De Rooij and Griswold, 2012; Nóbrega et al., 2009; Schulz et al., 2010). Similar to other anamniotes, in fish spermatogenesis occurs in cysts that are considered to be formed when a single spermatogonium is completely surrounded by the cytoplasmic projections of one or two Sertoli cells (McClusky, 2012; Pudney, 1993). In all vertebrate species already investigated, the cells resulting from differentiating mitotic divisions of single spermatogonia remain interconnected by cytoplasmic bridges that synchronize the developmental processes among the members of the same germ cell clone (Batlouni et al., 2009; Bosseboeuf et al., 2013; Loir et al., 1995; Pudney, 1995). The total number of germ cells in a cyst is greatly increased by the mitotic activity of spermatogonia, resulting, for instance, in ~750 and ~1360 early spermatids from one type A undifferentiated spermatogonia (A_{und}) respectively in Nile tilapia (Matta et al., 2002; Vilela et al., 2003)

Type A undifferentiated spermatogonia

These undifferentiated spermatogonia are isolated cells and also the largest germ cells in the fish testis.

A_{und1} A_{und2}

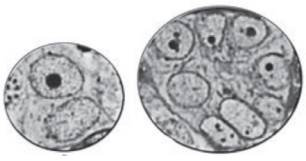
Morphological characteristics

- ✓ large nucleus with one or two conspicuous nucleoli,
- ✓ low nuclear heterochromatin,
- ✓ irregular nuclear envelope,
- ✓ large amount of perinuclear nuage (containing ribonucleoproteins and RNA),
- ✓ high density of mitochondria close to the nucleus and these mitochondria are surrounded by smooth endoplasmic reticulum.

Note: Two different types of A_{und} spermatogonia are found in zebrafish[1,2] and Nile tilapia[2] testis: A_{und1}; and A_{und2}. However, it is not known if these cell types represent two different phenotypes (e.g. during different phases of the cell cycle) of the same cell type, or if they are indeed distinct cells.

Type A differentiated spermatogonia

These differentiated spermatogonial cells are present in cysts with two to eight germ cells with incomplete cytokinesis (cytoplasmic bridges) after mitoses.



A_{diff} (2 cells) A_{diff} (8 cells)

Morphological characteristics

- ✓ nucleus showing one or more nucleoli,
- ✓ small amount of heterochromatin,
- ✓ round to oval nucleus with regular envelope,
- ✓ large cytoplasmic volume containing no or very little nuage.

Type B spermatogonia early

These spermatogonial cells are present in cysts with 16 or more germ cells, depending on the number of mitotic cell cycles (generations).

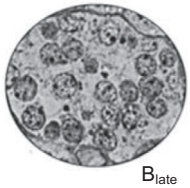
B_{early}

Morphological characteristics

- ✓ decreased cell size and nuclear volume,
- ✓ nucleus showing two or more nucleoli,
- ✓ high amount of heterochromatin,
- ✓ oval to round nucleus,
- ✓ smaller cytoplasm volume than in previous cells.

Type B spermatogonia late

These spermatogonial cells represent the latest generations prior the formation of primary spermatocytes.



B_{late}

Morphological characteristics

- ✓ usually round nucleus that are smaller than in the early type B spermatogonia,
- ✓ heterochromatin reaches the maximum density,
- ✓ round nucleus,
- ✓ cytoplasm volume smaller than in previous cells.

[1] Leal et al. 2009; [2] Schulz et al., 2010.

Fig. 1. Main morphological characteristics presented by fish spermatogonia, evaluated by high resolution light microscopy. Micrographs of different spermatogonial generations of Nile tilapia are shown, at the same magnification, in the left column. Type A undifferentiated spermatogonia (A_{und}), type A differentiated spermatogonia (A_{diff}), type B early spermatogonia (B_{early}), and type B late spermatogonia (B_{late} spermatogonia). Bar = 5 μm.

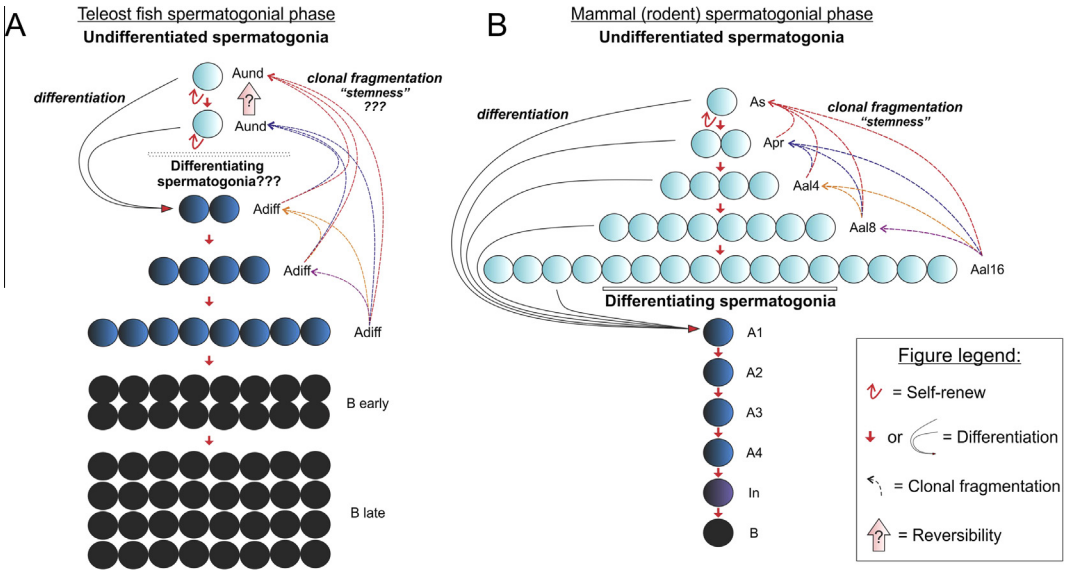


Fig. 2. Hypothetic scheme of the spermatogonial kinetics in fish based on the data available so far in the literature and proposed scheme of spermatogonial kinetics and stem cell renewal in rodents according to Nakagawa et al., 2010. In fish (A), the identity of A_{und} and A_{diff} is not well established and more molecular and functional studies are still necessary to elucidate if the so called "differentiating spermatogonia" are in fact already compromised with differentiation or if they can return to a more undifferentiated state (stemness capacity) through clonal fragmentation or functional reversibility. In rodents (B), these important physiological aspects are better characterized. Undifferentiated (A_{und}), single (A_s), paired (A_{pr}) and aligned ($A_{al4,\ 8,\ 16}$) type A spermatogonia; differentiated (A_{diff} and A_1, A_2, A_3, A_4) type A spermatogonia; intermediate spermatogonia (In); and type B spermatogonia (B).

and zebrafish (Leal et al., 2009). Because the spermatogenic process does not differ among vertebrates, the total number of spermatids per cyst in each fish species is related to the number of type A and type B spermatogonial generations (previously known respectively as primary and secondary spermatogonia (Billard, 1982, 1986; Grier, 1981; Pudney, 1995). According to the literature, the number of spermatogonial generations is considered to be species-specific and phylogenetically determined (reviewed in Nóbrega et al., 2009; Schulz et al., 2010). There is little information regarding specific morpho-functional or molecular characteristics of the spermatogonial cells in teleosts. Therefore, despite the confusing literature regarding their nomenclature, these cells are mostly identified by their size and morphological characteristics (Leal et al., 2009; Matta et al., 2002; Nóbrega et al., 2009; Schulz et al., 2005, 2010; Vilela et al., 2003).

In an attempt to standardize fish spermatogonia nomenclature and also to make it possible to compare them with better investigated mammalian species such as laboratory rodents (i.e. rats, mice, and hamsters); in fish these cells were morphologically classified by Schulz and colleagues (2010) as A_{und}, type A differentiated spermatogonia (A_{diff}), and type B spermatogonia (B early and B late). A_{und} spermatogonia are believed to self-renew and to give rise to type A_{diff} germ cells and, according to the literature (Lacerda et al., 2013b; Nóbrega et al., 2009; Schulz et al., 2010), different subtypes of A_{und} could be observed in Nile-tilapia and zebrafish. Also, as it will be approached in another section of this review, probably A_{und} present preferential location in the fish testis parenchyma.

Since the number of spermatogonia inside the spermatogenic cyst may reflects their extent of differentiation in a linear manner it is hypothesized that A_{diff} present a reduced potential for self-renewal if compared to A_{und} even though these cells still share some morphological characteristics (Fig. 1). In Nile-tilapia and zebrafish, A_{diff} are present in cysts with two to eight germ cells (Leal et al., 2009; Schulz et al., 2010). The principal morphological features that distinguish A_{und}, A_{diff}, B_{early} and B_{late} fish spermatogonia are presented in Fig. 1.

Although the point of no return to stemness is still debated (Nagano and Yeh, 2013), it is considered that fish and mammals type B spermatogonia are irreversibly committed to differentiation (Figs. 1 and 2) (Schulz et al., 2010). In mammals (De Rooij and Russell, 2000) and fish (Schulz et al., 2005), type B spermatogonia divide more rapidly than the type A spermatogonia. For instance, in African catfish a ~5-fold higher mitotic index of type B over type A spermatogonia is observed (Schulz et al., 2005). As already mentioned, there are several generations of type B spermatogonia and this number is considered to be species-specific. Therefore, these cells are present in cysts with 16 or more germ cells that are defined according to the number of mitotic cell cycles/generations prior the differentiation into primary spermatocytes (Schulz et al., 2010).

3. Spermatogonial kinetics and SSC renewal

In contrast to mammals, in which several different generations of germ cells are found together in the seminiferous epithelium, forming germ cells associations called stages (Hess and França, 2007; Leblond and Clermont, 1952; Muciaccia et al., 2013), in fish testis, Sertoli cells form cysts that envelope a single, clonally and hence synchronously developing group of germ cells deriving from a single spermatogonium. Up to date, as illustrated in Fig. 2A and considering only morphological criteria, type A_{und} spermatogonia (putative SSCs) in fish are single cells, i.e. not connected by cytoplasmic bridges to clone members. In mammals, (Nakagawa et al., 2010; Nagano and Yeh, 2013; Yoshida, 2012) (Fig. 2B), undifferentiated spermatogonia (A_{und}) consist of single cells, pairs, and chains of 4, 8, or 16 cells that are called Asingle (A_s), Apaired (A_{pr}), and Aaligned (A_{al}) spermatogonia, respectively. However, the recent model of spermatogonial physiology/kinetics suggested for fish (Fig. 2A) (reviewed by Schulz et al., 2010) is similar to the scheme proposed by Huckins (1971), Oakberg (1971) for mammals, in which the single cells among the As, Apr, Aal spermatogonia are the "actual" SSCs, implying that the formation of a pair (A_{pr}) constitutes the first step to differentiation.

It is assumed that fish spermatogonial differentiation always involve mitotic activity and therefore are not influenced by other germ cell association (called stages) as found in mammals. Particularly in laboratory rodents, most of the chains of A_{al} spermatogonia differentiate into the first type of "differentiating" spermatogonia during the epithelial stages VII–VIII (near to spermiation). Thus, these so-called differentiated cells are usually divided into ~6 generations [types A_1, A_2, A_3, A_4, Intermediate (In) and type B (B) spermatogonia] that divide synchronously in particular epithelial stages and primary spermatocytes are formed from their last division (Fig. 2B). In teleosts, more studies are still needed to accurately establish the spermatogonial kinetics.

In the Chondrichthyan fish (dogfish, *Scyliorhinus canicula*), Bosseboeuf and colleagues (2013) described the A_{und} and A_{diff} spermatogonial cells and their kinetics. In this recent study, similar to mammals and very different from teleosts investigated up to date, the group of A_{und} included A_s and large clones of spermatogonia (up to 512 cells). It means that the A_{und} spermatogonia in dogfish could divide 4 to 5 times more than in mammals and only after that they would give rise to differentiating (A_{d1}) and differentiated cells (A_{d2}, A_{d4}, A_{d8}). As an illustration of this order of magnitude, the total number of spermatids per cyst in the spiny dogfish (*Squalus acanthias*) is about 32,000 (McClusky, 2005), which is approximately 20 to 40-fold higher than that observed for Nile tilapia (Matta et al., 2002; Vilela et al., 2003) and zebrafish (Leal et al., 2009).

In mammals, to maintain the number of SSCs and to ensure a continuous production of A_{diff}, renewal of A_{und} clones is necessary. In rodents, Huckins (1971), Oakberg (1971) postulated that the spermatogonial self-renewing is entirely made up by divisions of A_s spermatogonia. Moreover, Huckins (1971) demonstrated that some pairs (A_{pr}) can split up in single cells (A_s) in a steady state situation. More recently, the theory that single cells (A_s) are the actual SSCs has been challenged by Nakagawa and collaborators (2010). Therefore, it is now suggested that not only A_s spermatogonia are SSCs and that in a steady state kinetics, pairs and chains can split up (clonal fragmentation), thus providing new single, pairs, and shorter chains as an alternative process (dotted lines in Fig. 2B) (Nakagawa et al., 2007, 2010). Thus, in order to maintain the epithelial/spermatogenic cycles, in rodents clonal fragmentation contributes significantly to the maintenance of SSCs numbers. To our knowledge, there is no such investigation in teleosts.

Although more studies are still necessary, Bosseboeuf and colleagues (2013) suggested for the first time that clonal fragmentation could occur in dogfish A_{und} population. Therefore, a question still remaining is if the considered A_{diff} in fish are able to return to a more undifferentiated stage through clonal fragmentation (dotted lines in Fig. 2A). In this particular context, more molecular and functional studies are required to evaluate the "stemness" capacity of teleost A_{und} cells. Nonetheless, as suggested for dogfish (Bosseboeuf et al., 2013) and rodents (Nakagawa et al., 2010; Yoshida et al., 2007), one could hypothesize that the so called A_{diff} in teleosts may be considered amplifying (progenitors) cells.

4. Spermatogonial cells plasticity

An aspect of great interest about fish A_{und} is related to their ability to transdifferentiate into oogonia after germ cell transplantation in a female recipient (Nóbrega et al., 2010; Okutsu et al., 2006; Yoshizaki et al., 2010). In this condition (Fig. 3A), in addition to X eggs the female recipients produced Y eggs and the ratio between males and females after mating was 3:1 (1 YY:2 XY:1 XX). Moreover, the resulting YY males were viable and fertile, suggesting that the X and Y chromosomes were at least functionally equivalent (Yoshizaki et al., 2010). This SSCs characteristic is very important for aquaculture when fish male production is desirable, particularly because the offspring of YY male is always male.

Another important finding is that fish spermatogonia can be originated from oogonia after their transplantation in a male recipient (Fig. 3B) (Okutsu et al., 2006; Yoshizaki et al., 2010). These newly-formed spermatogonia were able to differentiate and to originate fertile sperm carrying only X chromosomes. After transplantation, the recipient fish were mated with a wild-type female

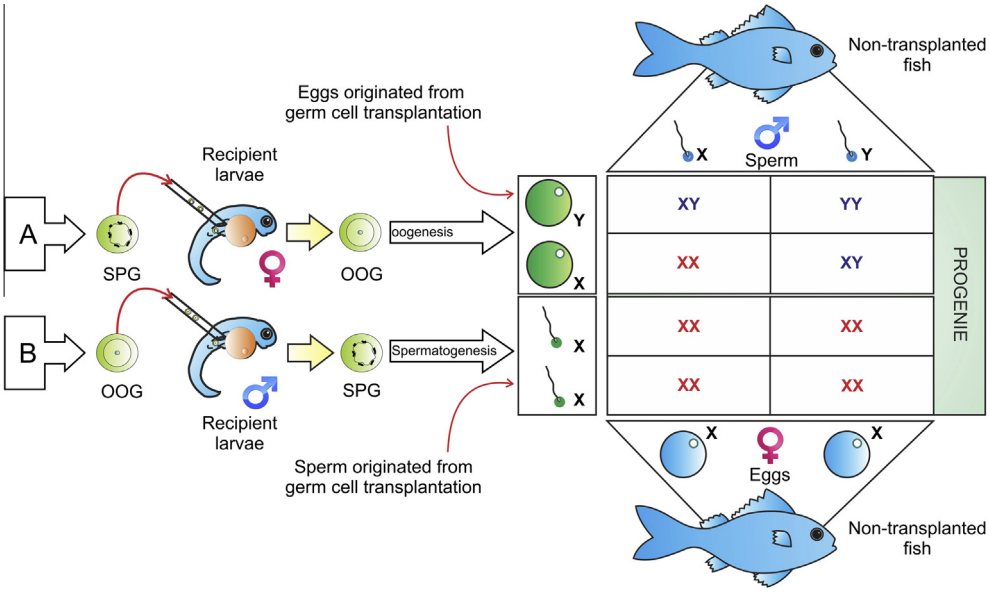


Fig. 3. Diagram illustrating the high level of sexual plasticity of fish spermatogonia and oogonia after germ cell transplantation in a recipient from the opposite sex. (A) As can be observed, the spermatogonia (SPG) possess the capacity to differentiate into oogonia (OOG) when transplanted into a female host. The newly-formed oogonia sustained oogenesis resulting in oocyte production containing X and Y chromosomes. After mating, this approach resulted in the offspring production with male-to-female sex ratio nearly 3:1. (B) On the other hand, the transplantation of oogonia into a male host was capable to originate spermatogonia that established spermatogenesis producing only sperm containing X chromosome. This later approach resulted exclusively in female offspring production. Taking together, these results indicated that in fish the host somatic microenvironment is crucial to determine the germ cell sex.

fish (XX) and all the offspring obtained were female. These results clearly indicate that, different from mammals, even after sexual maturity both fish type A spermatogonia and oogonia (probably germ stem cell) possess a high sexual plasticity. Moreover, the sex of fish germ cells is determined solely by the somatic microenvironment, rather than being cell autonomous (Yoshizaki et al., 2010).

5. SSCs distribution and niche

In some invertebrates (Sheng and Matunis, 2011) as well as in many mammalian species (Campos-Junior et al., 2012; Chiarini-Garcia et al., 2001; Costa et al., 2012; Yoshida et al., 2007), it is already established that the SSCs are preferentially located in specific areas of the testis called "niche". This special microenvironment may be controlled by the physical, cellular and molecular factors that regulate self-renewal, quiescence, and commitment to differentiation, sustaining spermatogenesis throughout the male reproductive life (Oatley and Brinster, 2012). Despite its crucial role for SSCs fate, the cellular and molecular composition of SSCs niche has remained elusive for almost all vertebrate species, including fish.

Considering the significant variation in the distribution of spermatogonia across fish species, the evaluation of their local microenvironment is quite important. According to Grier (1981), there are two different patterns of spermatogonial distribution in the teleost testis. The (i) restricted spermatogonia distribution, in which spermatogonia are located exclusively in the distal region of the germinal compartment (near to the tunica albuginea) and the (ii) unrestricted spermatogonial distribution, where these cells are spread along the germinal compartment throughout the testis. This latter pattern is typical for instance of Cypriniformes, Characiformes, and Salmoniformes (Parenti and Grier, 2004). However, an intermediate pattern also appears to exist between restricted and unrestricted spermatogonial distribution, such as that observed in Nile-tilapia (Vilela et al., 2003), in which A_{und} are the main spermatogonial cells type observed close to the tunica albuginea (Schulz et al., 2010).

Analyzing the topographical distribution of A_{und} in zebrafish (Nóbrega et al., 2010), that presents unrestricted spermatogonial distribution, we recently observed that, similar to mammals (Campos-Junior et al., 2012; Chiarini-Garcia et al., 2001; Costa et al., 2012; Yoshida et al., 2007), most of these cells are located in regions of the seminiferous tubules that are adjacent to the interstitial compartment (Nóbrega et al., 2010). Moreover, using testes of transgenic zebrafish expressing EGFP in endothelial cells (*fli:egfp*), A_{und} were often found in close association to blood vessels surrounding the seminiferous tubules (Nóbrega et al., 2010). Suggesting that the SSC niche also occurs in different fish species, preliminary results from our laboratory suggest that in both African catfish and Nile-tilapia A_{und} preferential location is observed (Fig. 4). Besides that, as is already well described in invertebrates (Sheng and Matunis, 2011) and chondrichthyes species (Bosseboeuf et al., 2013), the restricted spermatogonia distribution would represent a spatially well-defined SSC niche, in which certain areas of the testis may be suitable for self-renewal whereas other ones are more pro-differentiation.

6. SSCs regulation

Similar to mammals, fish SSCs survival, self-renewal, and differentiation result from a combination of several intrinsic and extrinsic factors (Miura and Miura, 2003; Skaar et al., 2011; Schulz et al., 2010). In the mammalian testis it is well established that, under FSH stimuli, Sertoli cells produces GDNF (glial cell line-derived

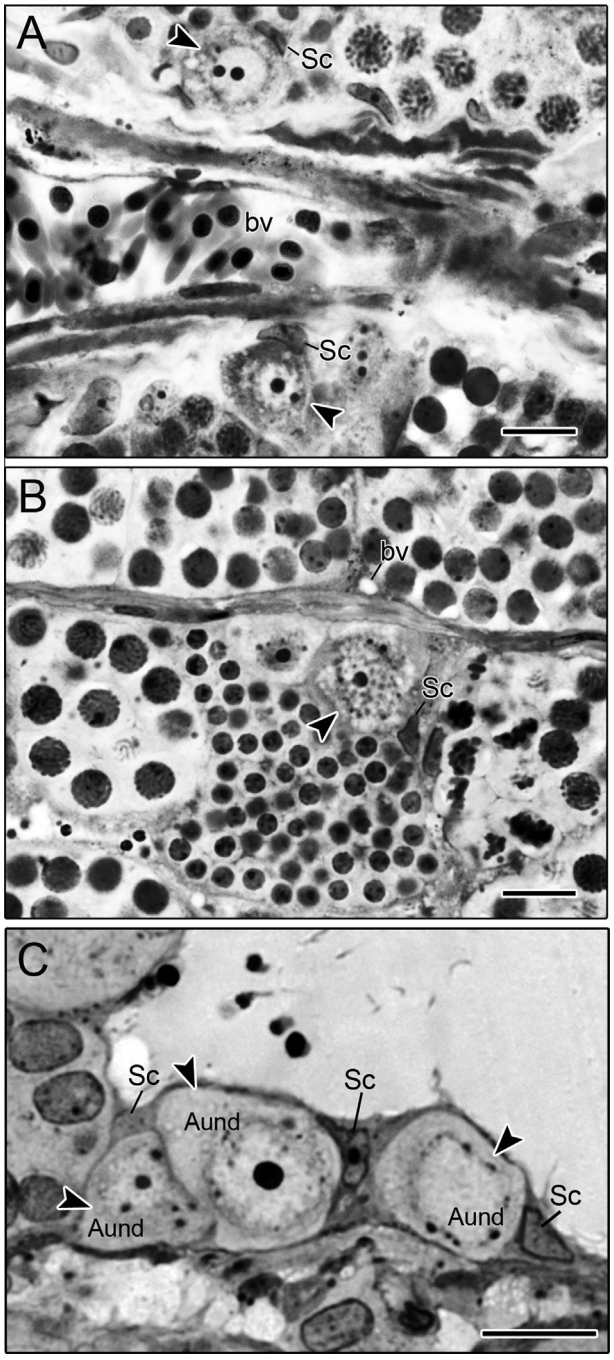


Fig. 4. Presumptive spermatogonial stem cell niche in fish according to the literature and recent data available for zebrafish (Nóbrega et al., 2010). As can be noted, suggesting the existence of SSC niche in other teleosts, in African catfish (A) and Nile-tilapia (B) testis, A_{und} spermatogonia (arrowhead) are preferentially located in regions of the seminiferous tubules adjacent to interstitium, particularly to blood vessels (bv). All A_{und} (arrowhead) are surrounded by Sertoli cells (Sc) that could also be in contact with different spermatogenic cysts (C). Scale bar = 10 μm.

neurotrophic factor) that binds the GFRA1 (GDNF family receptor alpha-1), which is expressed exclusively by A_{und} promoting its self-renewal and maintenance (Hofmann et al., 2005; Campos-Junior et al., 2012; Costa et al., 2012). Suggesting that this very important signaling pathway could also occurs in fish, the expression of Gfra1 in Nile-tilapia (Lacerda et al., 2013b), dogfish (Bosseboeuf et al., 2013) and rainbow trout (Nakajima et al., 2014) A_{und} spermatogonia were recently reported. However, because functional studies using specific fish GDNF have not been conducted, the role of this factor in this vertebrate class is not yet

elucidated. Nonetheless, *in vitro* studies (Kawasaki et al., 2012) showed that cocktail of recombinant mammalian growth factors containing GDNF, insulin-like growth factor 1 (IGF-1), and β fibroblast growth factor (bFGF), enhances the proliferation of zebrafish spermatogonia in a similar manner to mammalian culture systems; whereas Shikina and Yoshizaki (2010) reported no effect of rat GDNF or rat GFRA1-Fc fusion protein on the proliferation of rainbow trout type A spermatogonia. Interestingly, a recent study clearly showed that rainbow trout *gdnf* is expressed in germ cell (from spermatogonia to spermatocyte) but not in Sertoli cell, indicating that this factor would not be secreted as a SSC niche factor in the rainbow trout testis, unlike in mammals (Nakajima et al., 2014). These results suggest that other Sertoli cell factors could regulate the SSCs behavior in fish. Moreover, the possibility that one Sertoli cell could be in contact with different spermatogenic cysts would suggest a cross-talk between different germ cells clones (Fig. 4C).

In the Japanese eel, the involvement of three Sertoli cell derived factors [platelet-derived endothelial cell growth factor (PD-ECGF), activin B and anti-Müllerian hormone (Amh)] in the regulation of spermatogonial fate was reported (Miura and Miura, 2003). Therefore, under the influence of 17β-estradiol (E2), PD-ECGF is produced stimulating type A spermatogonia self-renewal (Miura and Miura, 2003), whereas 11-Ketotestosterone (11-KT) stimuli leads to the production of activin B by Sertoli cells, stimulating the proliferation of type B spermatogonia without initiating meiosis (Miura and Miura, 2003). In contrast, the Amh is involved in the inhibition of type A spermatogonia differentiation and in the blockage of type B spermatogonia proliferation (Miura et al., 2002, 2003, 2007). Furthermore, a recent study reported that recombinant eel growth hormone (GH) induces spermatogonial proliferation in a testis culture system, an effect that was independent of the production of steroid hormones or IGF-1 (Miura et al., 2011).

The induction of rainbow trout spermatogonia proliferation by IGF was first observed by Loir and Le Gac (1994) in an *in vitro* system. Later, Loir (1999) also demonstrated the potency of FSH, GH, IGF-1, and FGF-2 in stimulating the proliferation of spermatogonia present in primary cultures of total testicular cells prepared from trout testes at different stages of testis maturation. In the Nile-tilapia *in vitro* studies showed that IGF and/or human chorionic gonadotropin (hCG) promote spermatogonial proliferation and the onset of meiosis in the presence of 11-KT (Tokalov and Gutzeit, 2005). In zebrafish, Skaar and collaborators (2011) showed that recombinant Amh reduces proliferation and prevents differentiation of A_{und}. However, in contrast to the results obtained for the Japanese eel (Miura et al., 2007), increasing doses of 11-KT did not change Amh mRNA levels significantly (Skaar et al., 2011). Also in zebrafish, higher levels of 11-KT were observed after busulfan treatment, leading to SSC commitment to differentiation in recipient testis after germ cell transplantation (Nóbrega et al., 2010). These later findings indicate that Leydig cells and/or their secreted factors play a significant role in fish spermatogonial differentiation (Nóbrega et al., 2010).

Using primary testis tissue culture, the direct effect of triiodothyronine (T3) on the zebrafish testis was also investigated by our research group (Morais et al., 2013). In this study, we observed that T3 binds their receptors α (thrα; expressed by Sertoli cells) and β (thrβ; expressed by Sertoli and Leydig cells), stimulating the proliferation of A_{und} spermatogonia and Sertoli cells that are considered the main SSC niche component (Morais et al., 2013). More recently, it was demonstrated that the inhibition of BMP (bone morphogenetic protein) type I receptor prevents zebrafish SSCs differentiation in culture and enhances germline transmission of the SSCs following transplantation into recipient larvae. These results suggest that BMP signaling is detrimental for fish SSC self-renewal and maintenance (Wong and Collodi, 2013).

7. Spermatogonia phenotypic markers

In fish, SSCs are considered the largest germ cell present in the testis and these cells have been characterized mainly by morphological criteria (Koulish et al., 2002; Miura et al., 2003; Nóbrega et al., 2010; Papah et al., 2013; Schulz and Miura, 2002; Vilela et al., 2003). Therefore, until recently the enrichment of type A spermatogonia population was based almost exclusively on cell size and granularity, using density gradient sedimentation (Lacerda et al., 2006, 2012; Yoshikawa et al., 2009; Wong et al., 2013) or flow cytometry (Kise et al., 2012). In this regard, the lack of specific molecular markers represented a great limitation to identify and isolate SSCs and, similar to mammals, in the last decade spermatogonial transplantation was the only functional bioassay to test the stemness capacity of putative SSCs in fish (Lacerda et al., 2010, 2012; Nagano and Yeh, 2013; Okutsu et al., 2006; Yano et al., 2008).

In contrast to fish, in mammals, mainly in mice and rats, several molecular markers (e.g. ID4, GFRa1, c-RET, Pou5f1, Plzf, Nanos2, Thy1, Uchl1, SSEA-1, ITGA6, ITGB1, ERBB3, and SNAP91) have been used to identify and investigate A_s spermatogonia and their early progenitors (Abid et al., 2014; Kolasa et al., 2012; Phillips et al., 2010; Zheng et al., 2014). The available phenotypic markers of spermatogonia in fish are relatively limited and listed in Fig. 5. These markers will be critically reviewed and discussed below.

The first investigation involving the identification of stage-specific molecules in fish germ cells was demonstrated through the production of an antibody against the C-terminal amino acid sequence of eel P45011b, that recognized only early stages of spermatogonia in the Japanese eel (*Anguilla japonica*) testis (Kobayashi et al., 1998). The expression of the antigen, named spermatogonia specific antigen-1 (SGSA-1), was observed in type A and early type B spermatogonia (considered nowadays respectively A_{und} and A_{diff}; Schulz et al., 2010). Similarly, immunoreactivity anti-SGSA-1 was also detected in type A spermatogonia of medaka (*Oryzias latipes*) and Nile tilapia (*Oreochromis niloticus*) (Kobayashi et al., 1998). Although the molecular identification of SGSA-1 has not been achieved yet, in a later study the expression of SGSA-1 was also observed in type A (A_{und}) and early type B (A_{diff}) spermatogonia of loach (*Misgurnus anguillicaudatus*) (Yoshikawa et al., 2009).

In order to establish molecular markers for distinguishing the different spermatogonial types in the rainbow trout testis, the gene-expression profiles of enriched fractions of type A and type B spermatogonia were compared using salmonid cDNA microarray (Yano et al., 2009). Since only the receptor notch1 homologue showed higher expression in the type A spermatogonia-enriched fraction, this molecule was considered a useful type A spermatogonia marker for this fish species (Yano et al., 2009). Subsequently, a novel cell surface marker, lymphocyte antigen 75 (Ly75, also called CD205) was predominantly detected in rainbow trout type A spermatogonia (Nagasawa et al., 2010). Furthermore, Ly75 amino-acid sequences were found to be highly conserved in various teleosts species (Nagasawa et al., 2010). Moreover, Ly75 was identified as a specific type A spermatogonia cell surface marker in the Pacific bluefin tuna (*Thunnus orientalis*) (Nagasawa et al., 2012). Recently, combining next-generation sequencing of RNA with microarray, heterogeneity was observed in rainbow trout type A spermatogonia transcriptome (Hayashi et al., 2012). *Tubulin alpha chain* homog was expressed in a subpopulation of A spermatogonia, but not in the entire population in rainbow trout testis (Hayashi et al., 2012). Similar pattern of phenotypic heterogeneity is also observed in mice A_{und} (Oatley and Brinster, 2012).

The expression of GFRA1 and Nanos2 proteins in the Nile tilapia testis was recently investigated (Lacerda et al., 2013b). These molecules, that are considered classical markers of rodents A_{und}, represent key regulators for the maintenance and modulation of SSCs

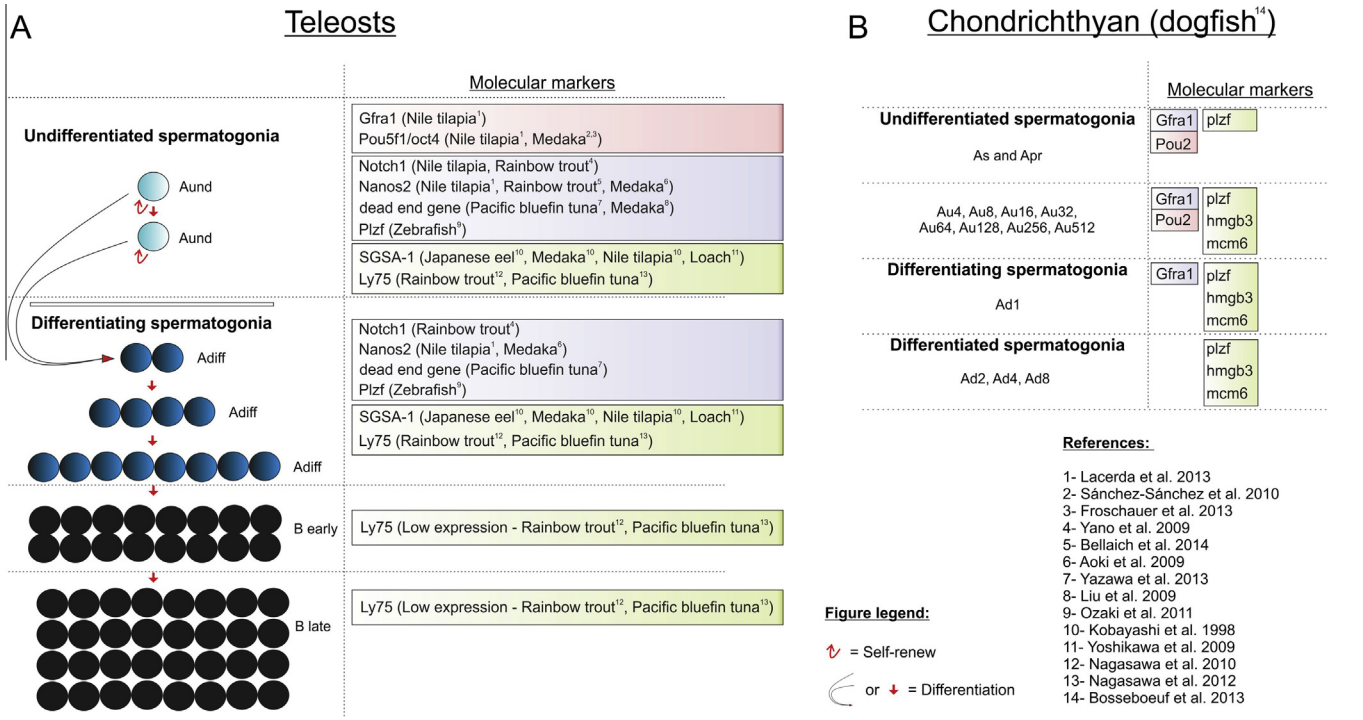


Fig. 5. Proteins expressed by undifferentiated, differentiating and differentiated spermatogonia in different fish species already investigated. In teleosts (A), the proteins expressed in undifferentiated (A_{und}) and differentiating (A_{diff} and B early/late) spermatogonia are listed in the right column. In the species (in parenthesis) investigated these proteins are listed within different box colors (pink, blue and green) according to their considered functional status (kinetics) indicated in the left column. In dogfish (B), the spermatogonial cell types (left column) and their respective protein expression (right column) are also shown within different box colors (pink, blue and green). As can be observed, for both fish groups the proteins in the pink boxes represent the ones exclusively expressed in the undifferentiated spermatogonia. The blue boxes include the proteins expressed in type A undifferentiated and differentiating spermatogonia. The green boxes contain the proteins that are normally expressed in all spermatogonial types. Undifferentiated (A_s, A_{pr}, $A_{u4\text{-}512}$), differentiating (A_{d1}), and differentiated ($A_{d2\text{-}8}$) type A spermatogonia.

self-renewal in mammals (Campos-Junior et al., 2012; Costa et al., 2012; Hofmann et al., 2005; Nagano and Yeh, 2013; Sada et al., 2012). Suggesting a conserved role for some molecules signaling in SSCs at the onset of vertebrate spermatogenesis, we demonstrated that the expression profile of Gfra1 in the Nile-tilapia SSCs is similar to that of rodents (Lacerda et al., 2013b). More specifically, it was found that in the adult Nile-tilapia testis Gfra1 was detected exclusively in single A_{und}, particularly on those found at the distal region of the seminiferous tubules located near to the tunica albuginea. In dogfish (Bosseboeuf et al., 2013), gfra1 mRNA was observed in A_{und} and, at a lower level, in early differentiating and differentiated spermatogonia (A_{d1}). In contrast to these findings, Gfra1 labeling was not found in *in vitro* studies using isolated spermatogonial cells in *Labeo rohita* (Panda et al., 2011).

Taking advantage of the excellent results obtained for Gfra1 (Lacerda et al., 2013b), we established a successful primary culture condition that allowed the Nile tilapia spermatogonia to expand their population for at least one month, while conserving their original undifferentiated (stemness) characteristics, measured by the presence of Gfra1 and other specific germ cells markers (vasa, Pou5f1/Oct4, and Sox2). Besides the evaluation of the maintenance of A_{und} phenotype, we performed a functional approach in which A_{und} Gfra1-positive cells were transplanted in the busulfan-treated adult Nile tilapia recipient testes, where they were able to form colonies and successfully develop (Lacerda et al., 2013b). These important findings convincingly advanced our knowledge on the identity and biology of fish SSCs and provided the establishment of a system that allowed *in vitro* amplification of fish SSCs.

Nanos2 expression in the mouse testis (Sada et al., 2012) is considered to be induced or maintained by the GDNF/GFRA1 signaling pathway. In zebrafish, at the early stages of sex differentiation, the expression of Nanos2 protein was observed in primordial germ cells (PGCs) (Beer and Draper, 2012); whereas oogonia and early spermatogonia were labeled by *in situ* hybridization in medaka fish (Aoki et al., 2009). Recently, Nanos2 expression was observed in isolated A_{und} as well as in cysts of germ cells considered A_{diff} in the adult Nile-tilapia testis (Lacerda et al., 2013b). In rainbow trout, *nanos2* expression was found to be restricted to a subpopulation of undifferentiated spermatogonia (less than 20%) present as isolated cells or in doublet in the immature and maturing testis. In contrast, *nanos2* transcripts was detected in all undifferentiated spermatogonia remaining in the spawning testis, suggesting that these cells would be considered putative SSCs in the trout testis (Bellaiche et al., 2014).

Pou5f1/Oct4 has been established as a specific pluripotency gene expressed in ES, ES-derived germ cells, PGCs and early germ cells of mammals and some fish species (Froschauer et al., 2013; Hong et al., 2004; Onichtchouk, 2012; Sánchez-Sánchez et al., 2010; Takehashi et al., 2012; Wang et al., 2011). Using immunohistochemistry we have observed the expression of Pou5f1 transcription factor in the testis of adult Nile-tilapia (Lacerda et al., 2013b), mainly in A_{und} located in the seminiferous tubules areas close to the tunica albuginea. Moreover, at 3 days of culture, Nile-tilapia A_{und} cells maintained the expression of Pou5f1/Oct4 (Lacerda et al., 2013b). Pou5f1/Oct4 is critically involved in the maintenance and self-renewal of undifferentiated and pluripotent cells (Baumann, 2013; Lengner et al., 2007), that could also be used for reprogramming (Okita et al., 2007). Therefore, we could speculate that, in comparison to those cells sited along the seminiferous tubules, the A_{und} located near to the tunica albuginea in the Nile-tilapia testis (Lacerda et al., 2013b) could be in a more undifferentiated or multipotent state. This assumption could explain the findings of Okutsu and colleagues (2006) showing that transplanted fish spermatogonial cells, probably SSCs (PGCs?), possess a high level of developmental plasticity and present sexual bipotency.

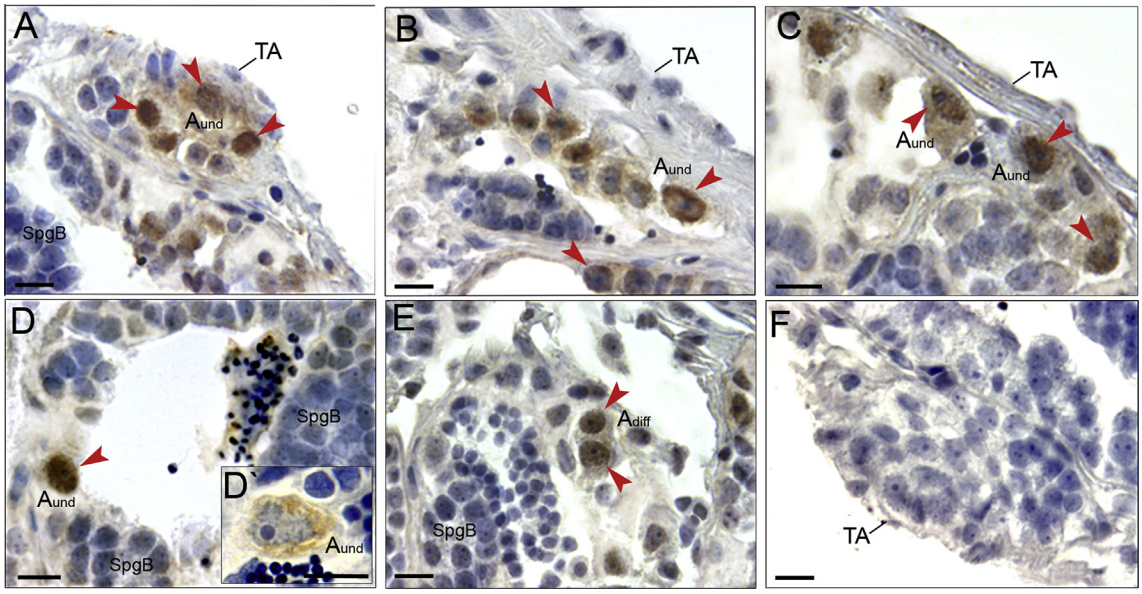


Fig. 6. Immunohistochemical localization of Notch1 protein in spermatogonial cells in the adult Nile tilapia testis. (A–C) Immunoreactivity was frequently found in clusters of single A_{und} (arrowheads) located in the distal area of seminiferous tubules near to the tunica albuginea (TA). (D–E) Notch1-positive spermatogonia (arrowheads) can also be observed along the seminiferous tubules as single cells (A_{und}; D), as well as in cysts containing 2 cells (A_{diff}; e). The inset (Ď) illustrates at higher magnification a typical A_{und} spermatogonia that express Notch1 protein. Type B spermatogonia (SpgB; A, D, and E) did not show any evident labeling. A negative control is shown in (F). Scale bar = 10 μm.

In mature dogfish testis (Bosseboeuf et al., 2013), Pou2/Pou5f1 transcript was found in A_{und}. Besides that, except for the cells considered A_s and A_{pr}, new spermatogonial markers such as hmgb3 (high mobility group box proteins 3) and mcm6 (mini-chromosome maintenance protein 6) were observed in all spermatogonia, representing therefore potential markers of more differentiated spermatogonia in this chondrichthyan species (Bosseboeuf et al., 2013).

Plzf is a transcriptional repressor found to be essential in the maintenance of mammalian SSCs (Buaas et al., 2004; Costa et al., 2012; Filipponi et al., 2007; Hobbs et al., 2010). In the adult zebrafish testis, specific antibodies raised against Plzf were used to determine the localization of this protein (Ozaki et al., 2011). In this study, immunohistochemical observations showed that Plzf was found in the nucleus of both type A and type B spermatogonia (A_{und} and A_{diff}, respectively according to Leal et al., 2009). In the dogfish testis (Bosseboeuf et al., 2013), Plzf mRNA was detected in A_{und} and A_{diff} as well as in meiotic cells and young spermatids. In a recent study, Bellaiche and colleagues (2014) showed that *pou5f1*, *plzf*, *nanos2* and *nanos3* mRNA are preferentially expressed in undifferentiated spermatogonia isolated form immature rainbow trout testis, and that, this cell population possess a high stemness capacity after germ cell transplantation.

The dead end (*dnd*) was previously identified in zebrafish as a gene encoding an RNA-binding protein essential for PGC migration and survival (Weidinger et al., 2003; Youngren et al., 2005). In contrast to the medaka fish (*Oryzias latipes*) (Liu et al., 2009), the Pacific bluefin tuna dead end (BFTdnd) gene was identified as a specific marker for A_{und} and A_{diff} (Yazawa et al., 2013) and the dnd-positive spermatogonia isolated from immature testis were able to form high number of colonies after transplantation in the recipient testis.

In a search of other potential markers for spermatogonial cells in fish, and based on the data available for rodents (Hayashi et al., 2001; Kostereva and Hofmann, 2008) and evidences from trout (Yano et al., 2009), we have been investigating the expression of the receptor Notch1 in the testis of adult Nile-tilapia. In these preliminary studies (data not published), using a commercial antibody anti-human Notch Homolog 1 (1:100; Lifespan Bioscience) made against a peptide sequence, that shows high similarity with Nile-tilapia notch homolog protein 1-like (XP_003451508.1), similar to Nanos2 we observed labeling in single A_{und} in the seminiferous tubules areas near to the tunica albuginea (Fig. 6). Suggesting that activated Notch1 might function to maintain the proliferation of germ cells as they progress through spermatogenesis, in rodents its expression starts before birth in gonocytes, increases as the germ cells proliferate and differentiate into type A and B spermatogonia, and peaks in spermatocytes (Hayashi et al., 2001; Kostereva and Hofmann, 2008). Also in rodents, via Sertoli cells (Cyp26b1 and Gdnf), it was shown recently that the NOTCH signaling orchestrates a dynamic balance between maintenance and differentiation of gonocytes (Garcia and Hofmann, 2013; Garcia et al., 2013). In humans, it is assumed that Notch-1 is specifically involved in germ cell differentiation (Hayashi et al., 2004).

8. Concluding remarks

The lack of SSCs molecular markers and the small number of SSCs in the testis represent a drawback to identify or purify SSCs in fish. In this regard, up to date the SSCs biology is still poorly known and the promising applications of SSCs in several valuable biotechnologies involving fish production are not yet well explored in this group of vertebrate. Moreover, the isolation or enrichment of A_{und} spermatogonia using specific molecular markers would significantly improve the efficiency of spermatogonial transplantation in fish, advancing also our knowledge on both basic and applied reproductive biology. In this context, in association with some good markers already available (ex: Gfra1, Pou5f1/Oct4, Notch1, and Nanos2), the establishment of a culture system of fish SSCs would allow to investigate important regulatory and functional aspects (ex: self-renewal and/or differentiation) of SSC biology in well-defined conditions. So far, the basic techniques for the long-term *in vitro* culture of spermatogonia have been established for Nile-tilapia (Lacerda et al., 2013b), zebrafish (Kawasaki et al., 2012; Wong and Collodi, 2013), rainbow trout (Shikina and Yoshizaki, 2010; Shikina et al., 2008, 2013) and medaka (Hong et al., 2004). Besides the aforementioned possibilities, such culture

system would allow the development of strategies to *in vitro* amplify SSCs of rare, endangered, or commercially valuable fish species (Shikina and Yoshizaki, 2010; Shikina et al., 2008), representing valuable tools for transgenesis and the development of new biotechnologies in fish production.

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